SUPPLEMENTARY INFORMATION

Phosphorylation of vascular endothelial cadherin is modulated by hemodynamic forces and contributes to the regulation of vascular permeability *in vivo*

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Supplementary Fig S1: pYVEC antibodies are specific for phosphorylated VE-cadherin and are suitable for in vivo staining

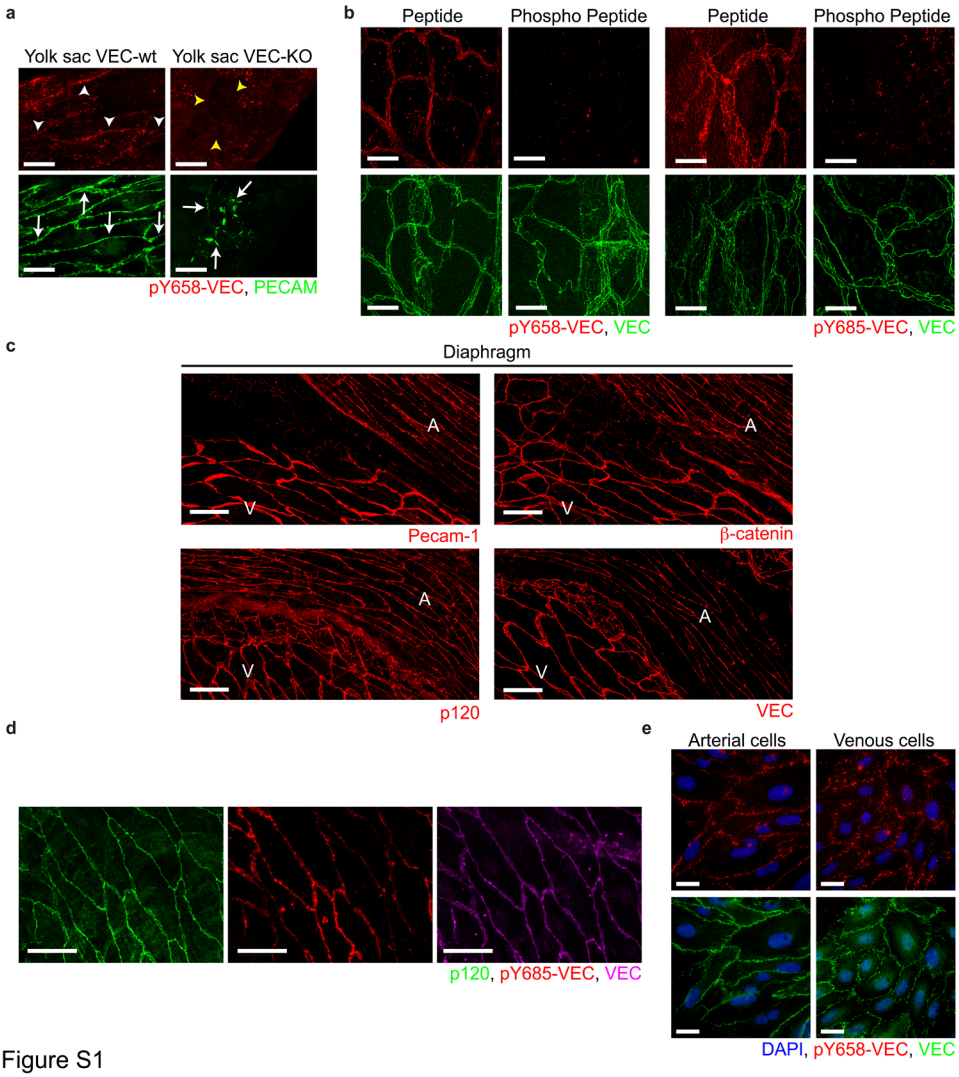

Figure S1

(a) Staining of mouse VEC positive and null embryo yolk sacs at 9.5 dpc with pY658-VEC antibody (n=3 embryos per group). EC were labeled by PECAM-1 (white arrows) and pY658-VEC (white arrowheads). In VEC null embryos, vessels are disorganized as previously reported [61], however PECAM-1 positive vessels are negative for pY658-VEC antibody staining (yellow arrowheads). Scale bars: 50 µm.

(b) To test for specificity, pY658- and pY685-VEC antibodies were pre-incubated with the respective phospho-peptide antigen (see Fig. 1a) or the same non-phosphorylated peptide. Pre-incubation with phosphorylated peptides eliminated antibody staining of the vessels. Conversely, the non-phosphorylated peptides were ineffective. Scale bars: 50 µm.

(c) Junctional markers such as PECAM-1, β-catenin, p120 and VEC are regularly stained at junctions of veins and arteries in vivo. Three different VEC antibodies were tested (see Methods) with superimposable results. Scale bars: 50 µm.

(d) In vivo staining of mouse diaphragm vein showed co-localization of Y685-VEC (red) and p120 staining (green). Scale bar: 20 µm

(e) Freshly isolated mouse arterial or venous EC were stained with pY658-VEC antibody. The antibody labeled intercellular junctions in a way similar to HUVEC (see Fig. 3a). No major difference in arterial and venous staining was detected. Scale bars: 20 µm.

Data are representative of at least 3 independent observations per group.

Supplementary Fig S2: VEC phosphorylation in vivo decrease upon bradykinin or histamine treatment.

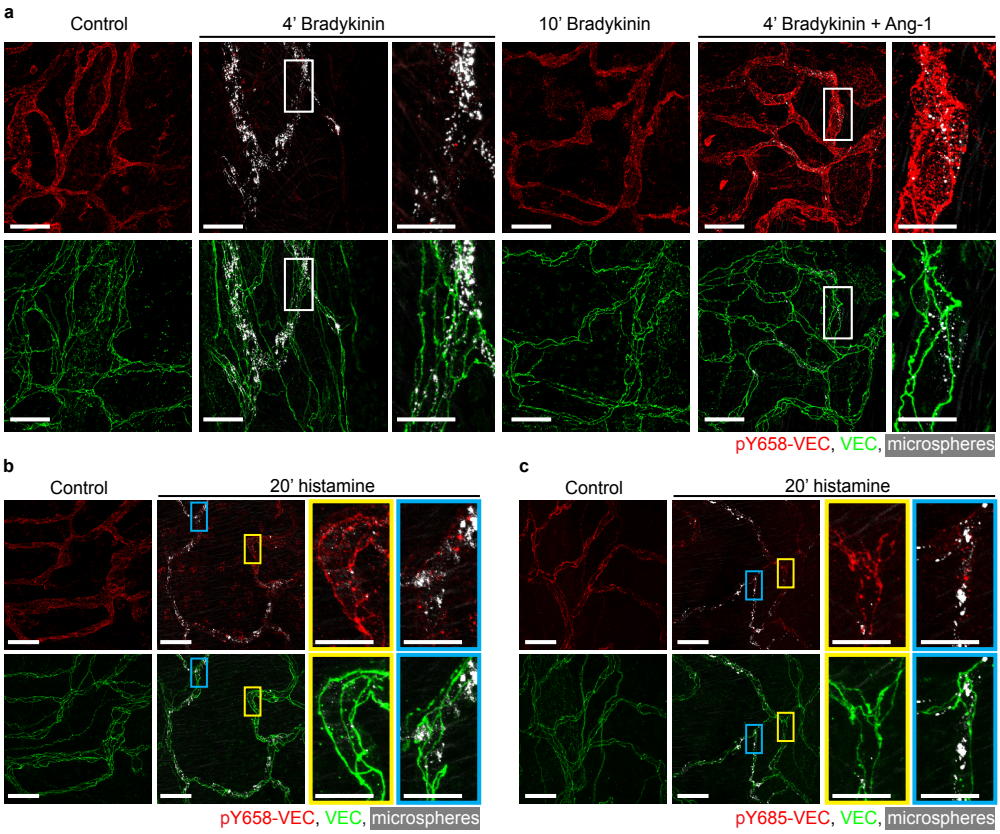

Figure S2

(a) In vivo staining with pY658-VEC antibody was comparable to that of pY685-VEC antibody (see Fig. 5a). pY658-VEC staining of veins was strongly decreased at 4 minutes after bradykinin treatment in vivo. This paralleled the increased permeability measured as microspheres leakage (white staining). At 10 minutes after bradykinin injection, VEC phosphorylation was restored. Ang-1 treatment abolished the effect of bradykinin on both venous permeability and VEC phosphorylation. Scale bars: 50 μm and 20 μm in magnified fields (n=10 mice for each treatment).

(b and c) Similarly to bradykinin, 20 minutes histamine treatment strongly decreased the staining of both Y658-VEC (b) of Y685-VEC (c) specifically in the areas affected by treatment, identified as the sites of microspheres leakage. Higher magnification of unaffected (yellow box) or affected (blue) region is shown. Scale bars: 50 μm and 20 μm in magnified fields (n=3 mice for each treatment).

Supplementary Fig S3: VEC does not dissociate from p120 upon bradykinin treatment.

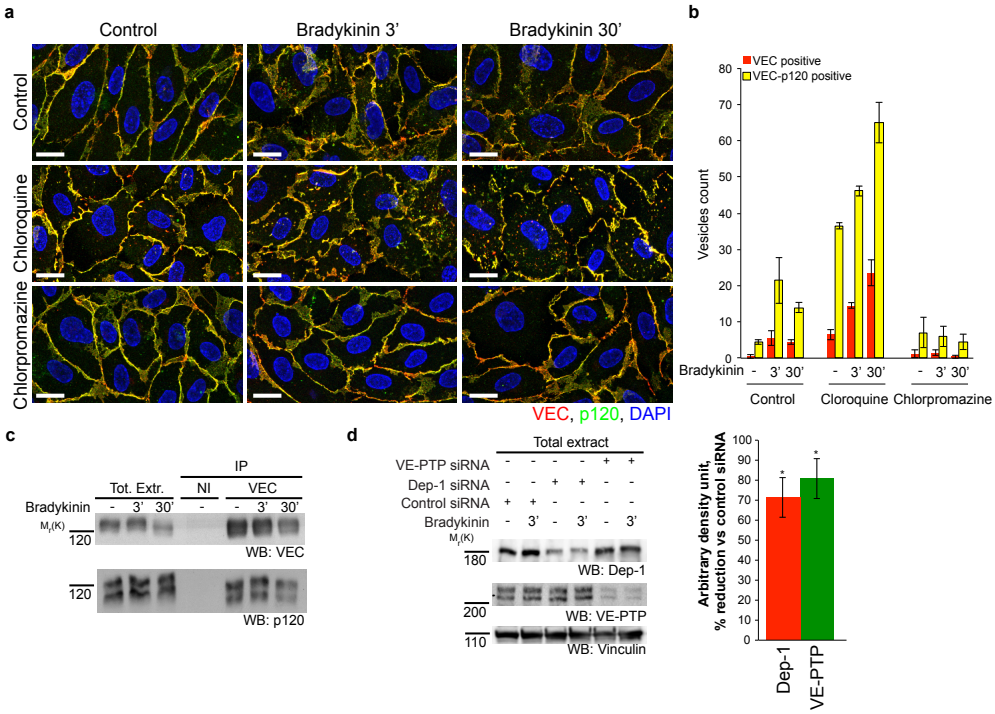

Figure S3

(a) Chloroquine treatment of cultured HUVEC induced a strong accumulation of intracellular VEC positive vesicles after 3 minutes of bradykinin treatment that was further increased at 30 minutes. p120 was found to co-distribute in part with VEC in internalized vesicles. Chlorpromazine (lower panels) inhibited both VEC and p120 internalization. Scale bar: 20 µm.

(b) Quantification of the internalization vesicles in (a). Vesicles showing co-localization of VEC and p120 or vesicles showing VEC staining only were counted separately. Bradykinin treatment was able per se to induce VEC internalization, however when the treatment was performed in combination with chloroquine the detection of internalization vesicles was strongly enhanced. Pretreatment of the cells with chlorpromazine completely block the bradykinin induced VEC internalization. Data are mean ± SEM of at least 50 cells analyzed.

(c) IP analysis of VEC after 3 minutes treatment with bradykinin show a comparable amount of p120 associated to VEC. Data shown are representative of at least 3 independent observations per group. (NI, not immune control).

(d) WB analysis on total extract and the quantification of band density shown that Dep-1 (red column) and VE-PTP (green column) expression were efficiently silenced upon siRNA treatment (> 70%). Data are shown as fold change versus control siRNA treated cells and shows mean ± SEM of three independent experiments.

* P<0.01 by independent two-tailed t-test assuming unequal variances.

Supplemental methods

Flow experiments

A custom-built flow chamber, consisting of two parallel plates made of Poly (methyl methacrylate) (PMMA) was used to apply uniform shear stress on HUVEC monolayers. While the lower plate was flat, a rectangular channel was engraved on the upper plate with an automatic milling machine.

HUVEC was grown to confluence on a patch of copolymer 2-norbornene-ethylene (COC)[66]. The patch was then placed on the lower plate and the upper plate was mounted to form a sealed channel of parallel-plate geometry.

The actual shear stress (τ) applied to the cells can be expressed in terms of volumetric flow rate (Q), medium viscosity (μ, $8.4*10^{-4}$ $Pa*s$)[67], width (w, 20 mm) and height (h, 0.3 mm) of the channel: $\tau = 6Q\mu / wh^2$. In our setup flow rates of 30 ml/minute, 90 ml/minute or 107 ml/minute were applied to obtain shear stress values of 3.5, 7, 14, 28, 42 or 50 dynes/cm^2, respectively. The indicated flow rates were controlled using a peristaltic roller pump (Model 66, Harvard Apparatus). Cells were fixed after 14 hours of constant flow.

PP1 (10 μM, Enzo Life Sciences) or DMSO was added to the culture media before the onset of flow. SU6656 (10 μM, Sigma) was added to the culture media 30 minutes before fixation. Chlorpromazine or chloroquine were added to the culture media 30 minutes or 3 hours (respectively) before bradykinin

treatment.

Co-localization data originate from multichannel fluorescence stacks collected using a Nikon-Ti (Nikon, Japan) wide-field microscope implemented with an Orca R2 CCD camera (Hamamatsu, Japan) and a 60x/1.2 NA water immersion objective (Nikon). For each stack a single value of the Pearson's coefficient (ranging between -1 and 1) was measured imposing a threshold value (calculated based on the algorithms by Costes et al.,[68]) for green and red channels using the 'co-localization analysis' section of Imaris (Bitplane).

Fluorescence Recovery after Photobleaching (FRAP)

HUVEC expressing mCherry-constructs were grown to confluence on μ-slide 8-well (Ibidi). Chlorpromazine and/or Cycloheximide (0.1 mg/ml) were added to the media 30 minutes before FRAP experiment. FRAP was performed on a spinning disk confocal microscope equipped with a FRAP PhotoKinesis unit (Nikon Eclipse Ti; UltraVIEW VoX; PerkinElmer) and driven by Volocity (Improvision) software as follow: 10 pre-bleach frames were acquired every 10 s, bleach was performed in a 90 μm^2 junctional area with 40 iterations using 488 nm (100%) and 561 nm (40%) laser line, post-bleach frames were acquired every 20 s for 10 minutes and every minute for the following 20 minutes. For each individual time-point, 7 z-planes with a step size of 0.4 μm were acquired

using a 60x/1.4 NA oil immersion objective and Hamamatsu emCCD Digital Camera.

Mean pixel intensity of 27 μm^2 area, corresponding to the central region of the bleached area, across the different time points. Residual fluorescence intensity of the bleached area immediately after bleaching (background signal) was subtracted from the fluorescence intensity of the bleached area over time; the resulting data were normalized on the average fluorescence intensity of the 10 pre-bleach frames.

The exponential kinetics of FRAP was analyzed using the "Curve fitting" ImageJ analysis tool for exponential recovery fitting ($y = a(1 - e^{-bx}) + c$). Recovery half-times ($\tau_{1/2}$) were calculated according to $\tau_{1/2} = -\ln(0.5)/b$.

Gelatine- glutaraldehyde crosslinking

To enhance endothelial cell adhesion, slides were coated with glutaraldehyde-crosslinked gelatin as follow. The culture supports were incubated 1 hour at RT with 1% gelatin followed by a crosslinking with 2% glutaraldehyde solution for 15 minutes at RT. The glutaraldehyde was replaced by 70% ethanol. After 1 hour, 5 washes with PBS followed by overnight incubation with PBS containing 2 mM glycine were performed. Before cell seeding, slides were washed 5 times with PBS.

Immunoprecipitation and Western blotting

For immunoprecipitation, individual capture antibody (3 μg/mg protein extract) was mixed with cell lysates for 1 hour at 4°C. Protein G plus Sepharose was added (20 μl) and mixed 1 hour at 4°C. Sepharose beads were washed 6 times, sample buffer was added and samples were boiled for 10 minutes. Immunocomplexes were resolved on SDS-polyacrylamide gels and subjected to Western blotting using standard protocols.

Buffered used for cells or tissues lysis were:

- JS buffer (20 mM HEPES pH 7.5, 1.5 mM $MgCl_2$, 5 mM EGTA, 150 mM NaCl, 1% Tx, 0.5% glycerol).
- RIPA buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Tx, 1% deoxycholic acid, 0.1% SDS, 2 mM $CaCl_2$).

Both buffers contained 1 mM Pefablock (Sigma), 1 mM NaF, 300 μM Sodium Orthovanadate (ACROS) and 600 μM H_2O_2.

Supplementary reference

61 Carmeliet, P. *et al.* Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. Cell **98**, 147-157 (1999).